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Award Number: DAMD17-00-1-0687

TITLE: Vascular Growth Factor Receptors as a Target for Auger
Electron Radiotherapy of Breast Cancer

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REPORT DATE: October 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20020429 133

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)**2. REPORT DATE**

October 2001

3. REPORT TYPE AND DATES COVERED

Final (1 Sep 00 - 1 Sep 01)

4. TITLE AND SUBTITLE

Vascular Growth Factor Receptors as a Target for Auger
Electron Radiotherapy of Breast Cancer

5. FUNDING NUMBERS

DAMD17-00-1-0687

6. AUTHOR(S)

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**8. PERFORMING ORGANIZATION
REPORT NUMBER****9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)****14. SUBJECT TERMS**

Breast Cancer

15. NUMBER OF PAGES

11

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

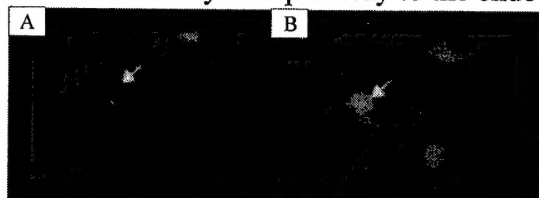
NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
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Introduction. Breast tumor is a aggressive and highly vascularized tumor in which angiogenesis is necessary to support growth..¹ Vascular endothelial growth factor (VEGF) is secreted by breast cancer cells² to stimulate angiogenesis through the over-expression of its receptors (VEGFR1 & 2) and proliferation of the vascular endothelial cells (VEC) of the surrounding tissue. In contrast, normal VECs express low level of VEGFRs. Strategies for targeting VEGFR to inhibit growth of breast cancer³⁻⁵ have been tested, but had limited success. On binding to its receptors, VEGF is internalized via the receptor-mediated endocytosis pathway to the endosomal compartment of the endothelial cells. Our preliminary



data using fluorescein-conjugated VEGF₁₆₅ showed that it was internalized into cytoplasmic vesicles inside human umbilical vein endothelial cells (HUVEC) (left panel A) and translocated to near the nucleus which formed a ring of fluorescence surrounding the nucleus (left panel B).

Therefore, the aim of the present project is to target the overexpressed VEGFRs on the endothelial cells of the breast tumor, to deliver lethal levels of Auger electron emitting radionuclide Indium-111 (¹¹¹In) into breast cancer associated VECs which could be an effective radiotherapy for breast cancer. To test this strategy, a novel recombinant protein was constructed by joining the N-lobe of human transferrin (nTF) through a flexible peptide linker (GGGGS)₃ to human VEGF (human VEGF₁₆₅ isoform) (nTF-VEGF). Native transferrin can bind 2 metal ions (including ¹¹¹In) in its 2 binding sites while nTF can bind one. ¹¹¹In has short effective range of less than 1 cell length, it will be radiotoxic only to cells that internalize it. Unlike the toxin-VEGF conjugate, the nTF-VEGF will have low immunogenicity in human due to the human origins of the two domains of nTF-VEGF. Also, the transferrin sequence increases the overall molecular weight of the fusion protein to slow its renal elimination and prolong circulation time in blood (longer half-life). The validity of our approach is provided by the data of Arora et al⁶ who used diphtheria toxin and VEGF₁₆₅ fusion protein to inhibit cell growth and protein synthesis in VEGFRs expressing Kaposi's sarcoma cell line (KS-Y1) implanted in immunodeficient mice and showed to have their growth inhibited by the conjugate. Such toxicity was restricted to proliferation of endothelial cells but not to the vascular smooth muscle cells.

Expression of the nTF-VEGF fusion protein. Biologically active VEGF_{7,8} and nTF₉ have been successfully expressed in yeast. Therefore, the yeast *Pichia pastoris* system was chosen to express the nTF-VEGF fusion protein. DNA Construction of nTF-VEGF. Transferrin (76 kDa) contains 2 symmetrical homologous lobes (N- and C- lobes). Each lobe is 38 kDa and can bind both ¹¹¹In and Fe with high affinity. VEGF exists in several isoforms. The 165 isoform is the most common and important for angiogenesis. Biologically active VEGF is a 46 kDa homodimer glycoprotein with the monomers covalently linked by disulphide bridges. The DNA construction and protein expression strategy for the fusion protein nTF-VEGF is based on the successful strategy used to express the diphtheria toxin-VEGF₁₆₅ recombinant protein by Arora et al⁶ but with a small modification. The diphtheria toxin was joined to the VEGF at the N-terminal, and the resulting fusion protein was shown to be toxic to VEGFRs-expressing cells. However, we included a highly flexible linker (GGGGS)₃ between the nTF and VEGF to facilitate flexibility between the nTF and VEGF domains (Fig. 1). This should ensure that the nTF-VEGF fusion protein can bind to the VEGFRs and be internalized.

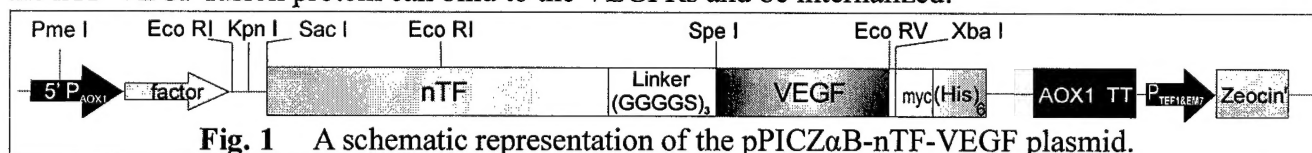


Fig. 1 A schematic representation of the pPICZαB-nTF-VEGF plasmid.

The DNA coding for nTF and VEGF₁₆₅ was in plasmids pBluescript SK-nTF and pCR2.1-VEGF respectively. Polymerase chain reaction (PCR) was done on these two plasmids using oligonucleotide

primers designed to remove nTF and VEGF leader sequences and stop codons, and to include cloning restriction sites. The nTF PCR DNA had Sac I cloning restriction site at the N-terminal, with the linker coding for the amino acid sequence (GGGS)₃ and a Spe I cloning restriction site at the C-terminal (see Fig. 1). nTF PCR DNA was digested with the restriction enzymes Sac I and Spe I to generate the cloning sites. The VEGF PCR DNA had a Spe I cloning restriction site at the N-terminal and a Eco RV cloning restriction site at the C-terminal (see Fig. 1). VEGF PCR DNA was digested with the restriction enzymes Spe I and Eco RV to generate the two cloning sites.

The nTF and VEGF DNA was ligated into pCR2.1 plasmid using standard molecular biology procedures to generate the pCR2.1-nTF-VEGF plasmid. Large scale DNA of plasmid pCR2.1-nTF-VEGF was prepared by transforming *E. Coli* DH5 α . The pCR2.1-nTF-VEGF plasmid has Ampicillin (Amp) resistance gene for selection, thus transformants were plated on Amp plates. Amp resistant *E. Coli* colonies were picked and plasmid DNA prepared (n=10). Restriction enzyme mapping was done on the plasmid DNA to identify bacterial colonies with the correct nTF-VEGF DNA. The plasmid with the correct restriction map then underwent DNA sequencing to confirm no sequence errors had been introduced during the cloning steps. The DNA fragment coding for nTF-VEGF was removed from the pCR2.1-nTF-VEGF plasmid using Kpn I and Xba I restriction enzymes and ligated into the *Pichia* expression vector pPICZ α B to generate the pPICZ α B-nTF-VEGF plasmid (see Fig. 1). This plasmid was transformed into *E. Coli* strain DH5 α . As the pPICZ α B-nTF-VEGF vector has the antibiotic Zeocin resistance gene for selection, Zeocin resistant *E. Coli* colonies were selected and plasmid DNA prepared (n=20). Restriction mapping of the plasmid DNA showed there was problem of stability of the pPICZ α B-nTF-VEGF vector in *E. Coli* DH5 α strain. Several other strains of *E. Coli* (INF α F', TOP10F' and TOP10) were tested for plasmid stability and TOP10 was found to be the best host for the plasmid. Therefore, plasmid pPICZ α B-nTF-VEGF was prepared from *E. Coli* TOP10 and analyzed by both restriction enzyme mapping (Fig. 2) and DNA sequencing to ensure no sequence errors were introduced.

Expression of nTF-VEGF fusion protein in *Pichia* yeast. The pPICZ α B-nTF-VEGF plasmid has a *Saccharomyces cerevisiae* α -factor prepro peptide secretion signal sequence at the start of the nTF-VEGF fusion protein to direct the secretion of the fusion protein into the medium (see Fig. 1). The C-terminal of the fusion protein has 2 tags: c-myc and 6x His for alternate methods of detection and purification.

Pichia yeast can metabolize methanol as carbon source using the enzyme alcohol oxidase which is encoded by the gene AOX1 and driven by P_{AOX1} promoter. It can be induced by methanol to very high levels. The pPICZ α B-nTF-VEGF plasmid has such methanol inducible AOX1 promoter upstream of the nTF-VEGF DNA (5' P_{AOX1}, Fig. 1) and thus this can drive the expression of the fusion protein to high levels when produced with methanol as carbon source. The pPICZ α B-nTF-VEGF plasmid was linearized by the restriction enzyme Pme I in the 5' P_{AOX1} promoter region (see Fig. 1) and used for integration into the *Pichia* genome via homologous recombination. The 'sticky' ends of the DNA at the Pme I restriction site are highly recombinogenic and interact directly with the homologous sequence on the yeast genome. In addition, restriction enzyme digestion within a region homologous to yeast genomic DNA increases the efficiency of integration at that homologous region.¹⁰ Therefore, it is highly likely that the plasmid integration would occur between the promoter in the *Pichia* yeast genomic AOX1 loci and AOX1 promoter on the linearized pPICZ α B-nTF-VEGF plasmid by a single crossover event. The most commonly used strains of *Pichia* (X33, GS115 and KM71H) were transformed with either the linearized plasmid pPICZ α B-nTF-VEGF (5 μ g) or pPICZ α B (5 μ g, control). The plasmids pPICZ α B-nTF-VEGF and pPICZ α B both contain the Zeocin resistance gene for selection. Zeocin resistant colonies of *Pichia* were selected on Zeocin plates for plasmid that had integrated into the *Pichia* genome. To detect nTF-VEGF DNA integrated in the *Pichia* genome, specific PCR amplification for DNA coding the VEGF (Fig. 3 lane 1) and nTF correspond to amino acid 166-331 (Fig. 3 lane 2) was done on genomic DNA of *Pichia* transformed with pPICZ α B-nTF-VEGF. The results showed correct-sized 503

and 498 b.p. PCR products respectively. These data showed the nTF-VEGF insert has integrated into the yeast genome. Such PCR products were absent when PCR on the genomic DNA of *Pichia* transformed with the control plasmid pPICZαB (data not shown).

The X33 and GS115 have intact AOX1 gene for methanol utilization (MUT^+) while KM71H has lost the AOX1 activity and it is slow methanol utilization (MUT^S). To determine the optimal growth conditions of *Pichia* yeast culture for expressing the fusion protein, the growth of *Pichia* transformants ($n=10$ for each strain) was compared on methanol and dextrose plates. X33 and GS115 *Pichia* transformed with pPICZαB-nTF-VEGF or pPICZαB plasmid grew normally on both methanol and dextrose plates and this showed the AOX1 gene was not disrupted by the integration of the plasmid. KM71H *Pichia* transformants grew slower on methanol plate than on dextrose plate, which showed the AOX1 gene remained disrupted. *Pichia* colonies that showed to have the pPICZαB-nTF-VEGF plasmid integrated were selected ($n=6$) and grown with methanol added to induce the promoter for protein expression. The yeast cultures were centrifuged, and the medium and cell pellet were analyzed for fusion protein content. SDS/PAGE (10%) under reducing conditions followed by Western Blot analyses using either polyclonal anti-VEGF (Fig. 4 lane 1), polyclonal anti-TF (Fig. 4 lane 2) or monoclonal anti-poly-His (Fig. 4 lane 3) showed a protein of about 65 kDa present in the medium. Such band was absent in the medium of *Pichia* transformed with control plasmid pPICZαB or inside the cell (not shown). These data showed the correct size fusion protein was successfully expressed in the *Pichia* yeast. Most of the fusion protein was present in the yeast media with no significant amount of the fusion protein detected in the yeast cell. The *Pichia* yeast KM71H strain was found to give the best expression level of the fusion protein.

Large scale purification of the nTF-VEGF fusion protein from the *Pichia* yeast culture.

To conduct in vitro endothelial cell culture inhibition studies and animal tumor growth inhibition studies, large amount of the fusion protein is required. The optimal expression level for nTF-VEGF in the medium of *Pichia* yeast KM71H strain was found to be 4 days after induction by methanol. The fusion protein was purified from the medium under native conditions. The yeast culture medium (10μl) was taken to analysis on the Western Blots using anti-TF (Fig. 5 lane 1) and anti-His (Fig. 5 lane 3) antibodies. The rest of the medium was mixed with the nickel-nitrilotriacetic acid (Ni^{2+} -NTA) metal-affinity chromatography resin (Ni^{2+} -resin) to allow the fusion protein to bind through the poly-His tag to the resin. The bound protein/resin was placed in a column and the fusion protein was eluted using phosphate buffer with imidazole (pH 8.0). The eluted fractions from the column (10μl) were also analyzed on the same Western Blots using anti-TF (Fig. 5 lane 2) and anti-His (Fig. 5 lane 4) antibodies. Then the fractions with the fusion protein were combined, dialyzed and concentrated using a Millipore M.W. 10,000 cut-off centrifugal filter unit and 10 μl was taken for analysis on 10% SDS polyacrylamide denaturing gel and stained with Coomassie-blue (Fig. 5 lane 5). These analysis showed that after Ni^{2+} column purification, only 1 major band was visible at about 65kDa corresponding to the nTF-VEGF fusion protein. Medium from the control *Pichia* culture (*Pichia* transformed with parent pPICZαB plasmid) showed no bands on Western Blots (figures not shown). These studies showed the correct fusion protein was expressed and purified. However, fusion protein yield was low at only 225 μg/litre of yeast culture.

The problem of low protein yield was overcome by carrying out optimization studies to increase the protein yield. The optimization studies included modifying the purification procedures using Co^{2+} metal affinity resin, with the bound proteins eluted at low pH instead of using imidazole displacement. Additional dialysis steps in acidic conditions were included to dissociate any prebound metal ions from the fusion protein nTF domain. Using the optimized conditions, the protein yield improved by more than 11 times to 2.625 mg/litre of yeast culture and HPLC analysis using gel filtration column showed the nTF-VEGF protein had a molecular weight of 130-150 kDa and the preparation was pure (Fig. 6). These HPLC data suggested that the nTF-VEGF fold into a dimer. SDS-PAGE gel under non-denaturing

conditions also showed the nTF-VEGF was 130-150 kDa (data now shown). Dimerization is essential for VEGF biological activity.

In vitro characterization of the purified nTF-VEGF fusion protein and biological activities.

nTF-VEGF fusion protein consists of two domains. nTF which binds ^{111}In and VEGF which binds to VEGF cell membrane receptors and is then internalized into the cell. The biological activities of both these domains were examined.

Biological activity of nTF domain. To examine the ability of nTF domain of the fusion protein to bind ^{111}In , purified nTF-VEGF (50 μg) was suspended in the assay buffer (pH 7)¹² and then mixed with 2 or 6 MBq ^{111}In and incubated at room temperature for 1 hour. Empty native transferrin (apoTF) (25 μg) was used as a control in these studies. Two procedures were used to analyze the mixture:

- 1) Column chromatography. The mixture was passed through gravity-flow size exclusion P2 column (BioRad). The protein was eluted with NaCl (150 mM). Fractions (100 μl) were collected and measured for radioactivity (Fig. 7). Unbound ^{111}In remained in the column. These analysis showed 33.58% of the 6MBq ^{111}In bound to the nTF-VEGF while 70.33% of the 6 MBq ^{111}In bound to the apoTF. Considering the difference in molecular size of nTF-VEGF and apoTF, the level of ^{111}In bound to them can be expressed by the specific activity of ^{111}In bound to the same number (or number of moles) of protein molecules. Thus, it was calculated that the nTF-VEGF and apoTF were labeled to specific activity of 142 mCi/ μmole and 355 mCi/ μmole respectively. These data showed the level of ^{111}In binding by the nTF-VEGF fusion protein was at least 40% compared to the native transferrin. The level of binding increased with higher specific activity of ^{111}In applied.
- 2) Thin-layer chromatography was performed on both labeled proteins, ^{111}In -nTF-VEGF and ^{111}In -apoTF, at pH 5 and pH 7. The results showed that the bound ^{111}In dissociated from both proteins at pH 5 (Fig. 8) and this result showed nTF-VEGF fusion protein retained the characteristic of metal ion binding and releasing as observed for the native TF.¹²

Biological activity of VEGF domain. To examine the VEGF domain of the fusion protein, proliferation assays by viability determination of HUVEC were done. HUVEC (5000 cells/assay) were plated and starved under 1:20 diluted growth medium for 1 day to remove the VEGF and other growth factors present in the culture. Medium with either nTF-VEGF or native VEGF (as control) was then added (ranging from 0 ng/ml to 100 ng/ml) to the assays and incubated for 3 days. A tetrazolium compound, which is bioreduced by viable HUVECs into a coloured formazan product, was added to each assay for colorimetric reaction. Comparing to the control assay of HUVEC incubating with medium with no nTF-VEGF or VEGF (0 ng/ml), the assays with 100 ng/ml of nTF-VEGF and VEGF included showed a 4.2 and 3.2 fold increase of growth of the HUVEC respectively (Fig. 9). The results showed that both nTF-VEGF and VEGF induced proliferation of HUVEC and the extent of induction was comparable to other investigations on proliferation of HUVEC induced by VEGF using thymidine incorporation, which ranged from 2 to 4 fold increase in proliferation.^{8,13}

^{111}In binding and HUVEC proliferation studies showed the nTF-VEGF fusion protein domains retained their biological activities as their two native proteins. Fluorescein isothiocyanate (FITC) labeling of nTF-VEGF, VEGF and TF has been done. Binding of these FITC-labeled proteins to the VEGFRs on HUVEC using flow cytometry, and studying their internalization into HUVEC are currently in progress to further characterize the in vitro biological activities of the nTF-VEGF fusion protein. These should be completed within the next four months. We request extension of this grant for a second year to conduct in vivo study using immunodeficient mice implanted with breast tumors to determine the effects of the fusion protein on the growth of breast tumors, its biodistribution and whether it is toxic to normal animal tissues. These will be carried out in the second year of the project.

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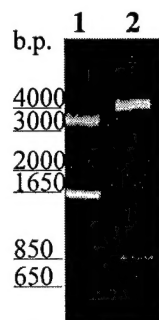


Fig. 2 Restriction enzyme map of the plasmid pPICZαB-nTF-VEGF. Restriction enzymes Kpn I+Xba I (lane 1) and Eco RI (lane 2) produced the correct-sized fragments of 3570+1602 b.p. and 4287+885 b.p. respectively.

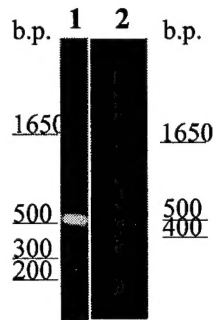


Fig. 3 PCR amplification of genomic DNA of Pichia transformed with pPICZαB-nTF-VEGF plasmid. Correct-sized DNA coding the VEGF (lane 1, 503 b.p.) and nTF (lane 2, 498 b.p.) were observed.

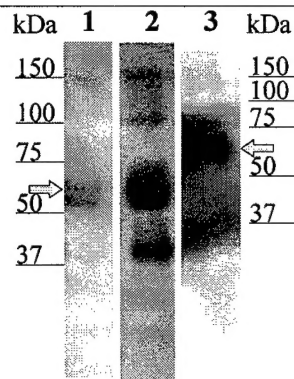


Fig. 4 Pichia KM71H transformed with pPICZαB-nTF-VEGF plasmid was grown and centrifuged. The medium was analyzed for fusion protein. SDS/PAGE (10% under reducing conditions) followed by Western Blot analyses using antibodies against VEGF (lane 1), TF (lane 2) or poly-His (lane 3) showed a protein of about 65 kDa present in the medium (arrow).

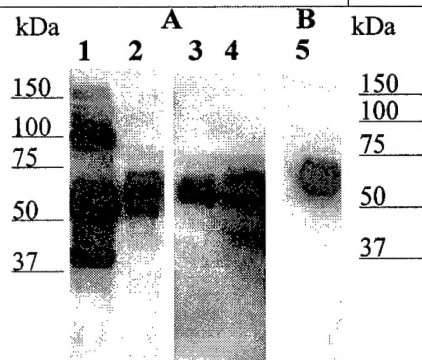


Fig. 5A Western Blots were done on the yeast culture medium using anti-TF (track 1) and anti-His (track 3) and showed the presence of the 65 kDa band. Eluted fusion protein from Ni^{2+} column was analyzed using Western Blots with anti-TF (track 2) and anti-His (track 4) showed the 65 kDa band was the dominant band. Fig. 5B Molecular weight analysis of fusion protein from Ni^{2+} column on 10% SDS/PAGE stained with Coomassie-blue showed only the 65 kDa band.

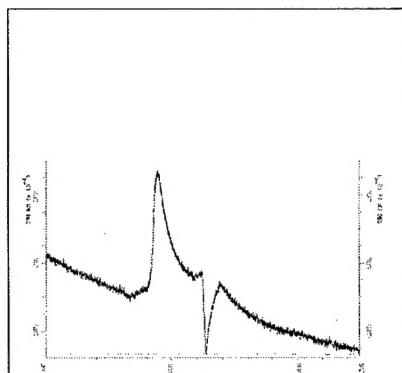


Fig. 6 Determination of purity, size and the heterogeneity of the conformers present in the purified nTF-VEGF fusion protein by HPLC with gel filtration column. The preparation of nTF-VEGF was pure and the fusion protein exists as a dimer of 130-150 kDa in size.

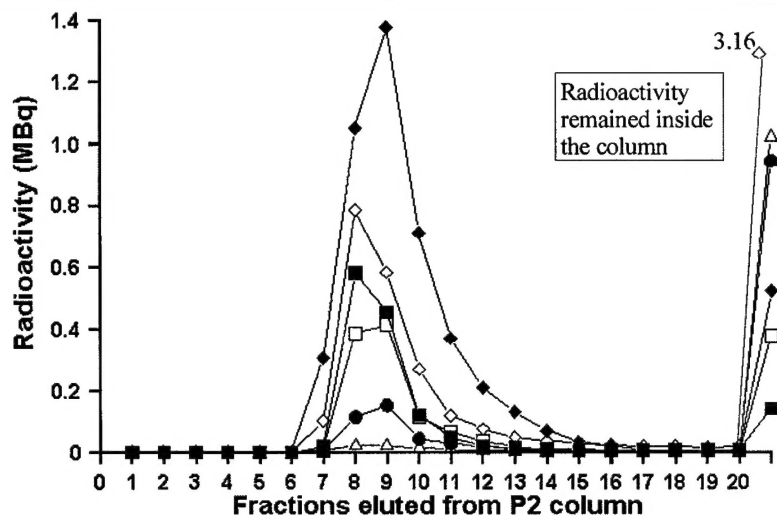


Fig. 7 ^{111}In binding assay of nTF-VEGF and apoTF. Mixtures of \square 2MBq ^{111}In /nTF-VEGF 50 μg , \blacksquare 2 MBq ^{111}In /apoTF 25 μg , \bullet 2 MBq ^{111}In /nTF-VEGF 25 μg , \triangle 2MBq ^{111}In , \diamond 6 MBq ^{111}In /nTF-VEGF 50 μg and \blacklozenge 6 MBq ^{111}In /apoTF 25 μg were incubated for 1 hour and passed through P2 size exclusion chromatography column. The labeled proteins were eluted at around fractions 8-9. The nTF-VEGF and apoTF were labeled to specific activity of 142 mCi/mole and 355 mCi/mole respectively. Thus, the level of ^{111}In binding by the nTF-VEGF fusion protein was at least 40% compared to the native transferrin.

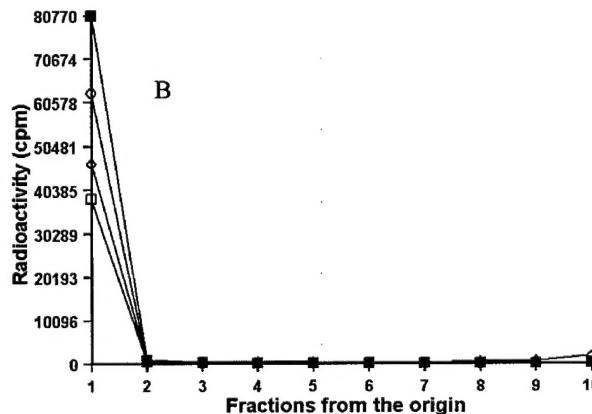
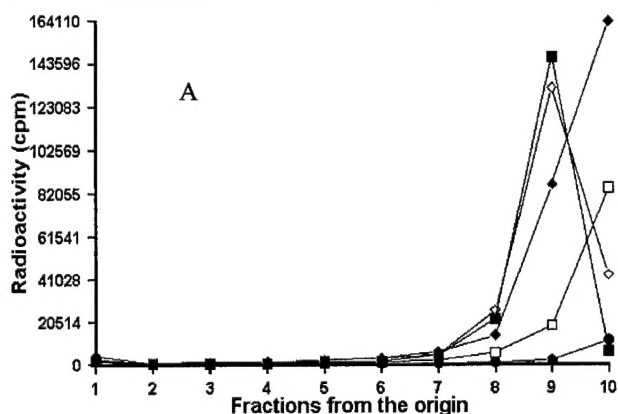


Fig. 8 Thin layer chroma-tography of the ^{111}In -labeled nTF-VEGF and TF eluted from the P2 size exclusion chromatography column. (A) pH 5 and (B) pH 7 sodium citrate were used as the carrier buffer. (Data point markers are the same as those described in legend of Fig. 7.) ^{111}In dissociated from both proteins at pH 5.

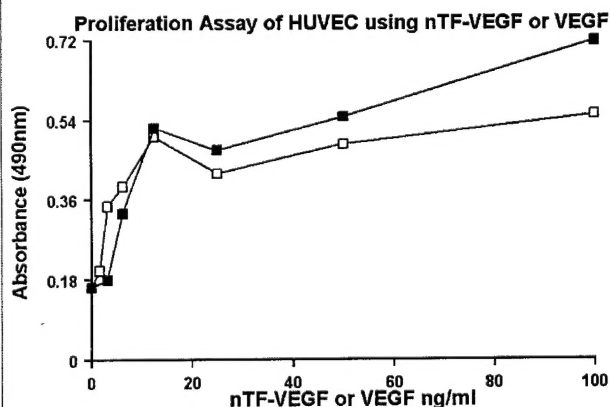


Fig.9 HUVEC proliferation assay. The HUVEC were starved for 1 day in 1:20 diluted growth medium. Medium with nTF-VEGF (\blacksquare) or VEGF (\square) was added and incubated for 3 days before colorimetric reagent was added and absorbance was recorded after 3 hours in 37°C. Each value is a mean of triplicate cultures. Both nTF-VEGF and VEGF showed to be able to induce growth of HUVEC. Comparing to no nTF-VEGF or VEGF was added (0 ng/ml), there was a 4.2 and 3.2 fold increase of growth when nTF-VEGF or VEGF (100 ng/ml) was added respectively.

